

AD _____

Award Number: DAMD17-02-1-0572

TITLE: Autocrine and Paracrine Control of Breast Cancer Growth by
Sex Hormone-Binding Globulin

PRINCIPAL INVESTIGATOR: William Rosner, M.D.

CONTRACTING ORGANIZATION: St. Luke's Roosevelt Institute for
Health Sciences
New York, New York 10019

REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

BEST AVAILABLE COPY

20040901 027

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Apr 2003 - 31 Mar 2004)	
4. TITLE AND SUBTITLE Autocrine and Paracrine Control of Breast Cancer Growth by Sex Hormone-Binding Globulin			5. FUNDING NUMBERS DAMD17-02-1-0572	
6. AUTHOR(S) William Rosner, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) St. Luke's Roosevelt Institute for Health Sciences New York, New York 10019 <i>E-Mail:</i> Wr7@columbia.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) <p>We propose that the expression of Sex Hormone-Binding Globulin (SHBG) by breast cancer cells is biologically regulated and this SHBG functions to alter the effects of estrogens within the breast cancer cell. We have shown that plasma protein sex hormone-binding globulin (SHBG) not only binds estrogens in plasma, but also is part of an estrogen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on breast cell membranes rather than the intracellular estrogen receptor (ER). The SHBG-R_{SHBG} complex is activated by an appropriate steroid hormone, eg. estradiol (E_2), (forming the new complex, E_2-SHBG-R_{SHBG}), to trigger a second messenger system to produce cAMP within minutes after steroid binding. We have shown that frozen sections of normal and cancerous breast cells stain with anti-SHBG antibodies and these same cells contain SHBG mRNA. Further, the well-known breast cancer cell line, MCF-7, contains both SHBG mRNA and SHBG protein. The expression of SHBG by breast cancer cells raises the important question of how local regulation of SHBG synthesis and secretion affects both sequestration of steroid hormones within the breast, and estrogen induced signal transduction at the cell membrane. The local synthesis of SHBG is consistent with an autocrine/paracrine role in breast cancer.</p>				
14. SUBJECT TERMS Hormones; estrogens; membrane receptors; cancer; steroids; agonists; antagonist; cell signaling; second messenger; transcription; SHBG;				15. NUMBER OF PAGES 16
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	10
References.....	11
Appendices.....	12

Progress report: "Autocrine and Paracrine Control of Breast Cancer Growth by Sex Hormone-Binding Globulin"

INTRODUCTION

We propose that the expression of Sex Hormone-Binding Globulin (SHBG) by breast cancer cells is biologically regulated and that this SHBG functions to alter the effects of estrogens within the breast cancer cell. We have shown that the plasma protein sex hormone-binding globulin (SHBG) not only binds estrogens in plasma, but also is part of an estrogen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on breast cell membranes rather than the intracellular estrogen receptor (ER) (1). The SHBG- R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2), (forming the new complex, E_2 -SHBG- R_{SHBG}), to trigger a second messenger system to produce cAMP within minutes after the steroid binds. We have shown that frozen sections of normal and cancerous breast cells stain with anti-SHBG antibodies and that these same cells contain SHBG mRNA. Further, the well-known breast cancer cell line, MCF-7, contains both SHBG mRNA and SHBG protein. The expression of SHBG by breast cancer cells raises the important question of how the local regulation of SHBG synthesis and secretion affects both the sequestration of steroid hormones within the breast, and estrogen induced signal transduction at the cell membrane. The local synthesis of SHBG is consistent with an autocrine/paracrine role for this protein in breast cancer.

To this end, we will conduct genetically and pharmacologically based studies to address our hypothesis. The second year has been devoted to three major studies, 1) generation and characterization of breast cell lines that inducibly or constitutively overexpress SHBG and condition their medium with secreted SHBG; 2) characterization of SHBG mRNA expression in breast cell lines; 3) characterization of SHBG protein expression in breast cell lines, and normal and tumor tissues. The cell lines will be used to investigate the effects of SHBG expression on selective stimulation of either the ER or the SHBG-receptor based pathways, and the effects of conditioned medium from these cell lines on cell growth in year three. The detailed pharmacologic approach that we will use will allow us to dissect the influence of estrogens on the two pathways of interest and further to ascertain how each contributes to the growth of breast cancer cells. We will also further characterize our exciting observation that novel SHBG mRNAs, containing different 5' sequences, are synthesized in MCF-7 cells.

BODY

We have made considerable progress in creating cell lines that overexpress SHBG, having now obtained all necessary reagents for their full characterization. We have new and potentially exciting evidence that in the MCF-7 cell line, SHBG is expressed as a novel isoform(s), different from the hepatically derived isoform that we have already overexpressed in MCF-7 and MDA-MB-231 cells. We have completed immunohistochemical and in situ analyses of SHBG protein and mRNA expression in breast cell lines, normal breast tissue, and breast tumors. We intend to further

characterize the novel SHBG isoforms that we suspect are being expressed in MCF-7 cells. If this latter finding extends to other breast cell lines, normal breast tissue and breast tumors, it opens the exciting possibility that the breast expresses SHBG isoforms with different functions than the hepatically derived SHBG isoform. Our studies planned for the upcoming third year of this grant will address these important questions.

1. Anti-human SHBG antibodies for Western blot analysis.

A major technical problem that we addressed this past year was identifying an antibody that would be suitable for Western blot analysis of cellular extracts. First, we tested all polyclonal and monoclonal antibodies that we have generated in house or have obtained from outside sources on HepG2 cellular extracts and purified SHBG protein from human serum. None proved useful for Western blot analysis. We next had custom polyclonal antisera generated against both the secreted form of SHBG (encoded by the cDNA used in these studies) and the alternative form of SHBG first described in the testis (2). Unique peptides, CLRPVLPTQSA and CFSRLRLTHPPRTW, corresponding to the respective SHBG isoforms, were synthesized and used to immunize rabbits. Affinity purified antisera were positive by ELISA assay, however they did not prove useful for Western blot analysis. Fortunately, at the beginning of 2004, we obtained a polyclonal antibody, WAK-S102-12-53 (WAK-Chemie, Steinbach, Germany), which is useful for Western blot analysis. As detailed below, we used WAK-S102-12-53 to confirm ELISA and PCR results on cell lines that inducibly or constitutively express various SHBG constructs at elevated levels.

2. Generation of M13S14, an MCF-7 cell line with inducible SHBG expression.

A major part of our effort this year was also devoted to creating appropriate MCF-7 and MDA-MB-231 cell lines for our functional studies on SHBG. The parental intermediate clonal cell line, MCF7-pVgRXR 13, had incorporated functional pVgRXR sequences (the PonA-inducible transactivator), showed no leakiness, and had a high degree of inducibility in control experiments. We stably transfected the inducible plasmids pIND/Hygro/SHBGsense and pIND/Hygro/SHBGantisense, and the control plasmid, pIND/Hygro into MCF7-pVgRXR 13, and isolated 24 individual hygromycin resistant clones from each transfection. After expansion of the 24 clones, DNA was prepared, and analyzed by PCR for uptake of SHBG sequences (or vector sequences for the control transfectants). Positive pIND/Hygro/SHBGsense clones were analyzed for induced SHBG expression by treating cells with 10 μ M ponasterone A, and measuring SHBG in cellular. PonA markedly induces SHBG in M13S14 and less markedly in M13S24, which also shows some basal leakiness (**Figure 1**). As expected, PonA did not induce SHBG expression in the vector control cell line, M13V7. We next plan to analyze candidate inducible antisense clones with WAK-S102-12-53.

3. Generation of MDA-MB-231 clonal cell lines that constitutively overexpress SHBG: pSHBG-FL and pSHBG-MP.

Unexpectedly, MDA-MB-231 cells were highly resistant to killing by the drug zeocin, including relatively large doses of up to 2mg/ml. Because we select for pVgRXR uptake with zeocin, we instead attempted to introduce pVgRXR into MDA-MB-231 cells by cotransfecting this plasmid with the vector pCMVFlag (described below), which contains

a selectable neomycin resistance gene. Transfections were performed using a 10:1 ratio of pVgRXR to pCMVFlag. We performed PCR analysis on 24 G 418 resistant colonies, and these all proved negative for pVgRXR, as did the pool of remaining colonies. We are currently repeating transfections using even higher ratios of pVgRXR to pCMVFlag.

As an alternative, we synthesized two additional constructs for evaluating the effects of constitutively expressed SHBG in MDA-MB-231 cells. The vector used for these experiments was pCMVFlag, a plasmid containing a CMV promoter upstream of an ATG start codon, immediately followed by three iterations of a Flag tag (4) sequence. We generated, 1) pSHBG-FL, a plasmid containing the full length SHBG cDNA sequence cloned immediately downstream of the Flag tags and in the same reading frame, and 2) pSHBG-MP, a plasmid containing SHBG cDNA lacking the 29 amino acid amino terminal leader sequence which, in the liver, is cleaved from the nascent protein before secretion. Thus, this Flag tagged protein mimics the mature, processed SHBG found in serum. However, since it lacks the leader sequence, we hypothesized that the SHBG-MP protein would remain inside cells. We expected that the Flag tag would allow us to specifically detect the expressed pSHBG-FL and pSHBG-MP proteins in western blots. In addition, the Flag tag would produce different sized SHBG proteins, distinguishable from endogenous SHBG.

We expected that SHBG-FL might be detectable in cells, but it would have the Flag tag cleaved along with the signal peptide prior to secretion. However, if the cleavage system was overloaded with SHBG-FL protein, we might also detect residual full length SHBG-FL protein. We expected that the SHBG-MP construct would remain inside cells because it lacked a leader sequence, and might serve to mimic the fate of absorbed SHBG from outside. The CMV promoter would direct constitutive expression of these proteins at elevated levels.

pSHBG-FL and pSHBG-MP were transfected into MDA-MB-231 cells. Following selection, 12 resistant colonies from each transfection were isolated and expanded. MDA-MB-231-MP7 was positive in Western blot analysis using the anti-Flag monoclonal antibody (**Figure 2A**), and cell extracts from 12 MDA-MB-231-FL clones have recently been prepared for analysis.

4. Generation of MCF-7 clonal cell lines that constitutively overexpress pSHBG-FL and pSHBG-MP.

We also transfected pSHBG-FL and pSHBG-MP into MCF-7 cells, and expanded 12 individual clones from each transfection. Three MCF-7-FL clones had elevated amounts of SHBG protein (ELISA). Western blot analysis showed that MCF-7-FL10 had high expression of the Flag-tagged SHBG construct (**Figure 2B**). Furthermore, this clone secreted non Flag-tagged SHBG (**Figure 2B**), demonstrating that the leader peptide is properly processed from Flag-tagged SHBG-FL, and that the mature processed protein is secreted just like the secreted form of SHBG in the liver. We have isolated 12 MCF-7-MP clones, and these will be ready for Western blot analysis shortly.

5. MCF-7 cells express novel SHBG isoforms- structural analysis of MCF-7 mRNA species.

The bulk of published data on SHBG gene expression at the mRNA level are based on work that was performed prior to the advent of PCR. It had been thought from early cDNA cloning experiments that two mRNA species are synthesized from the human SHBG gene (2-3). The first is the secreted form of SHBG, made in the liver. This 8 exon-long species is the isoform we have overexpressed above. The second SHBG isoform was originally described in the testis, and apart from a recent publication that shows it to be expressed in human sperm (5), it remains very poorly characterized. This isoform uses an upstream promoter and therefore has different first exon sequences. Early cDNA studies suggested that this isoform has exon 7 sequences spliced out.

During the past year, we obtained data suggesting that SHBG gene expression in MCF-7 cells is different from the picture described above. We prepared first strand cDNA from total cellular MCF-7 RNA using oligo dT primers. Using primers that amplified exon 5-8 sequences, we unexpectedly generated three RT-PCR transcripts (**Figure 3**). Sequence analysis revealed that the largest transcript contained faithful splicing of exon 5,6,7, and 8 sequences. The intermediate transcript was missing all of exon 7 sequences. The smallest transcript was missing both exon 6 and 7 sequences.

Using primers that amplified exon 2-8 sequences, we generated four RT-PCR transcripts (data not shown). The largest transcript has a predicted size of contiguous exon 2-8 sequences. The intermediate sized transcripts are of sizes consistent with the splicing out of exon 7 and exons 6 and 7, respectively. The smallest transcript is of a size lacking exons 5, 6, and 7; however, these results are awaiting sequence analysis to confirm the size predictions. Thus, in MCF-7 cells, we have evidence that at least four different SHBG mRNA species are synthesized. These mRNAs would encode proteins that differ both in size and at their carboxy termini.

We were unable to generate RT-PCR transcripts from MCF-7 cells using secreted SHBG exon 1 specific primers (**Figure 4**). As expected, HepG2 RT-PCR samples were positive for the secreted SHBG exon 1 transcript (6). This is a very important finding, suggesting that a different upstream promoter is being utilized in MCF-7 cells. We plan to test whether MCF-7 cells are using the alternative upstream promoter, first described in the testis. We have designed PCR primers specific for testis SHBG isoform first exon sequences. Total RNA from human sperm will serve as a positive control for the testis isoform. It also remains possible that MCF-7 cells use a novel promoter well upstream of the testis promoter (3). Once we have a clearer picture of these new SHBG transcripts, we will be able to determine their hypothetical protein sequences, and we will be able to design appropriate experiments to address their function.

6. Immunohistochemical analysis of SHBG protein expression in human breast cell lines and effects of steroid binding to SHBG on membrane localization.

SHBG is visualized clearly in the cytoplasm, but not the nucleus of MCF-7, MDA-MB-231, and 184-B5 cell lines (**Figure 5**). Note that most of the heavy staining in MCF-7 cells is perinuclear (**Figure 5A**). MCF-7 cells stain more intensely than the other lines

and also (less apparent) show staining in a greater fraction of cells than do MDA-MB-231 or 184-B5 cells (**Figure 5B and 5C**). Nonpermeabilized MCF-7 cells showed membrane staining for SHBG, consistent with secretion of SHBG followed by binding to R_{SHBG} (**Figure 6A,B**). How these observations fit with our finding that alternative SHBG transcripts are synthesized in MCF-7 cells is unclear at the moment, but is consistent with a protein that retains its membrane binding site. MDA-MB-231 cells showed little if any membrane binding in nonpermeabilized cells (data not shown).

The current model for steroid signaling through R_{SHBG} includes binding of steroid-free SHBG to R_{SHBG} as an initial step. Biochemical studies have shown that SHBG, prebound to steroid, is unable to bind to R_{SHBG} . To test this model in vivo using MCF-7 cells (known to possess R_{SHBG}), we asked whether specific steroids that bind to SHBG could displace it from the membrane (7). MCF-7 cells treated with purified SHBG showed a marked increase in membrane staining, and in intracellular SHBG (**Figure 6C**). Cells treated with SHBG that had been preincubated with 2-methoxyestradiol (2MeOE₂), a steroid that binds tightly to SHBG, showed a marked decrease in membrane staining (**Figure 6D**). These results are consistent with prior biochemical findings (8). We then tested the effects of MCF-7 cells treated with SHBG that had been preincubated with dihydrotestosterone (DHT) or testosterone (T) (both of which are tightly bound to SHBG) (**Figure 6E and 6F**). None of the steroids added in conjunction with SHBG appeared to have any effect on intracellular SHBG (permeabilized cells) (data not shown).

Although MDA-MB-231 cells have cytoplasmic SHBG, none is associated with the cell membrane, either from endogenous SHBG or added SHBG. These data indicated an absence of R_{SHBG} in these cells (Data not shown).

7. Immunohistochemical analysis of SHBG and in situ analysis of SHBG mRNA expression in normal human breast tissue and breast tumors.

In normal breast tissue, staining for SHBG is most intense in epithelial cells, predominating in luminal epithelial cells (**Figure 7A**). Because there is a substantial concentration of SHBG in plasma, the staining seen in sections of human breast could result from simple diffusion of SHBG from plasma with subsequent cellular uptake. To address this issue, we examined sections for SHBG mRNA expression by in situ hybridization. SHBG mRNA (**Figure 8A**) is detected in similar areas, also predominating in luminal epithelial cells. Similar results were seen using adjacent sections (data not shown). Although SHBG protein and mRNA appear to colocalize in cells, normal breast tissue is heterogeneous, with areas of intense staining and areas of zero to light staining.

SHBG protein expression is abundant in cancerous areas of the breast (**Figure 7B**) with strong staining seen in periglandular epithelium. Epithelial cells, both normal and cancerous, stain strongly for SHBG mRNA, and there is minimal staining in stromal cells (**Figure 8B**). These observations are consistent with a model in which SHBG is synthesized and secreted primarily by epithelial cells and binds to R_{SHBG} on epithelial and/or stromal cells.

We have been delayed in our functional analyses because of the difficulty we had in obtaining a suitable anti-human SHBG antibody for use in Western blot experiments and the unexpected resistance of MDA-MB-231 cells to zeocin. We now have the reagents and most of the cell lines, including M13-FL10 which secretes copious amounts of SHBG into the medium. We have purposely devoted much time to what we consider a complementary and highly important aspect of our study, namely characterizing the new SHBG mRNA transcripts that we have discovered in MCF-7 cells. The structures of these species should tell us whether the proposed studies mimic only the effects of serum-derived SHBG on breast cells, or include the effects of endogenously synthesized SHBG.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of the anti-human SHBG polyclonal antibody, WAK-S102-12-53, for use in Western blot analysis.
- Generation of M13S14, an MCF-7 subclone engineered to inducibly express the hepatic, secreted form of SHBG. Generation of M13V7, a control MCF-7 subclone.
- Synthesis of pSHBG-FL and pSHBG-MP, flag tagged full length and mature processed SHBG constructs for constitutive expression. Generation of 12 candidate MDA-MB-231-FL clonal cell lines, and MDA-MB-231-MP7 that constitutively expresses a stably incorporated flag tagged mature processed SHBG construct. Generation of MCF-7-FL10 which constitutively expresses a stably incorporated flag tagged full length SHBG. Generation of 12 candidate MCF-7-MP cell lines.
- Demonstration that elevated amounts of SHBG can be made in MCF-7 and MDA-MB-231 cells, and that MCF-7 overexpressing cells secrete SHBG. Demonstration that the flag tagged mature processed SHBG protein is stable and remains intracellular.
- MCF-7 cells express multiple SHBG mRNA species, including forms that retain exons 2-8, and those that splice out exons 7, 6+7, and perhaps 5, 6, and 7. RT-PCR results suggest MCF-7 cells express novel SHBG isoforms that lack the exon 1 sequences present in hepatically derived SHBG. MCF-7 cells could use the testis promoter, or a novel upstream promoter.
- SHBG protein is expressed in the cytoplasm of the MCF-7, MDA-MB-231, and 184-B5 cell lines. In MCF-7 cells, SHBG protein is strikingly perinuclear.
- Immunohistochemical results demonstrate SHBG binding to the membrane of MCF-7 cells, but not MDA-MB-231 cells, suggesting the latter lack R_{SHBG}. MCF-7 membrane binding can be displaced by preincubation of SHBG with 2-methoxyestradiol, T, and DHT.
- In normal breast tissue, staining for SHBG is most intense in epithelial cells, predominating in luminal epithelial cells. SHBG mRNA colocalizes to these same cells, suggesting that in vivo, endogenously synthesized SHBG mRNA is translated and expressed. Normal breast tissue itself appears heterogeneous, with areas of intense staining and areas of zero to light staining.

- SHBG protein expression is abundant in cancerous areas of the breast with strong staining seen in periglandular epithelia. Strong staining for the SHBG mRNA was seen in epithelial cells as well as in carcinoma cells infiltrating the stroma adjacent to the epithelial cells.

REPORTABLE OUTCOMES:

1. Fourth International Symposium on Hormonal Carcinogenesis. Valencia, Spain, June 21-25, 2003

Poster presentation and book chapter:

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

Scott M. Kahn^{1,2}, Daniel J. Hryb¹, Atif M. Nakhla¹, Saeed M. Khan, Nicholas A. Romas², and William Rosner¹

Departments of Medicine¹ and Urology², St. Luke's/Roosevelt Hospital Center, and College of Physicians and Surgeons, Columbia University, New York, N.Y

2. Third International Meeting- Rapid Responses to Steroid Hormones. Florence, Italy, Sept. 12-14, 2003

Poster presentation:

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

Scott Kahn^{1,2}, Daniel J. Hryb¹, Atif M. Nakhla¹, Nicholas A. Romas², William Rosner¹

Departments of Medicine¹ and Urology², St. Luke's/Roosevelt Hospital Center, and College of Physicians and Surgeons, Columbia University, New York, N.Y. 10019

CONCLUSIONS:

We have shown that MCF-7 and MDA-MB-231 cells can be used to produce the hepatically secreted isoform of SHBG. From our initial RT-PCR results, we expected that this was the major isoform expressed in breast cells. However, based on our newer RT-PCR results, this may not be the case. If we confirm that a novel SHBG isoform(s) is expressed in MCF-7 cells and in normal breast epithelial cells in general, this could change our prior hypothesis of how allelic deletions of the SHBG gene locus could contribute to breast cancer. The distinct perinuclear immunohistochemical staining of SHBG seen in MCF-7 cells is striking, and raises new questions about SHBG function. It is also unclear whether all or just some of the transcripts we have detected are translated. Now that we have a useful polyclonal antibody for Western blot analysis, we will be able to address these and other questions. If novel isoforms are the endogenously expressed species in the breast, we will use a similar overexpression and downregulation strategy to ascertain their functions. The strategy that we outlined for the hepatically secreted isoform is still useful for further characterizing the still unclear function of R_{SHBG} signaling in breast cells.

REFERENCES:

1. Kahn SM, Hryb DJ, Nakhla AM, Romas NA, Rosner W. 2002 Sex hormone-binding globulin is synthesized in target cells. *J Endocrinol.* Oct;175(1):113-20.
2. Gershagen S, Lundwall A, Fernlund P. 1989 *Nucleic Acids Res.* Nov 25;17(22):9245-58.
3. Hammond GL, Bocchinfuso WP. 1996 Sex hormone-binding globulin: gene organization and structure/function analyses. *Horm Res.*;45(3-5):197-201.
4. Knappik A, Pluckthun A. 1994 An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. *Biotechniques.* Oct;17(4):754-61.
5. Selva DM, Hogeveen KN, Seguchi K, Tekpetey F, Hammond GL 2002. A human sex hormone-binding globulin isoform accumulates in the acrosome during spermatogenesis. *J Biol Chem.* Nov 22;277(47):45291-8.
6. Mercier-Bodard C, Baulieu EE. 1986 Hormonal control of SBP in human hepatoma cells. *J Steroid Biochem.* Jan;24(1):443-8.
7. Hryb DJ, Nakhla AM, Kahn SM, St George J, Levy NC, Romas NA, Rosner W. 2002 Sex hormone-binding globulin in the human prostate is locally synthesized and may act as an autocrine/paracrine effector. *J Biol Chem.* Jul 19;277(29):26618-22.
8. Hryb DJ, Khan MS, Romas NA, Rosner W. The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J Biol Chem.* 1990 Apr 15;265(11):6048-54.

APPENDICES:

Figure 1. SHBG overexpression is induced by PonA in M13S14 cells

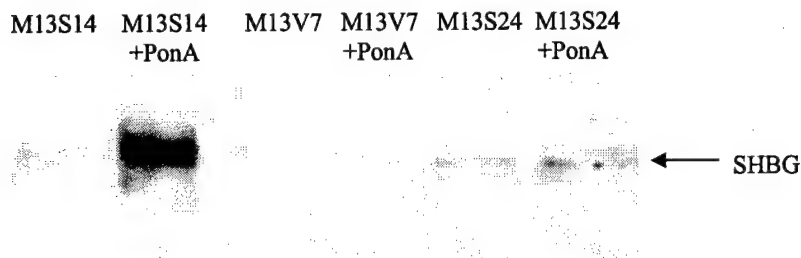


Figure 1: PonA induces SHBG protein expression in MCF-7 cells stably transfected with pVgRXR (a plasmid encoding the PonA-activatable transactivator), and the inducible construct, pIND/Hygro/SHBGsense (a plasmid that expresses the secreted form of SHBG in response to the activated transactivator). M13S14, M13V7, and M13S24 cells were plated in duplicate in 6 well dishes at 75% confluence. After incubating for 48 hours, cells in one well were exposed to 10uM PonA for 24 hours, while control, unexposed cells were mock treated with solvent. Total cellular protein was prepared and analyzed by Western blot. Low amounts of SHBG were visible on the original film in the M13V7 lanes. Parental M13 cells showed similarly low expression of endogenous SHBG, on a par with untreated M13V7 cells, and were not affected by PonA treatment

Figure 2. Western blot analysis of SHBG overexpression in MB-MDA-231 and MCF-7 clonal cell lines

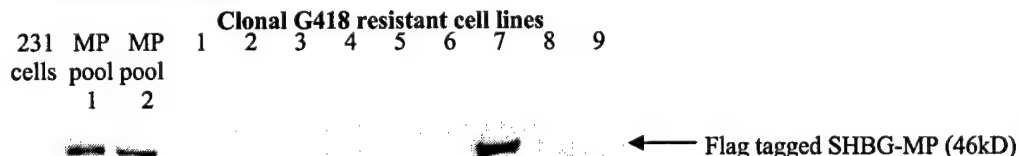


Figure 2A: MB-MDA-231-MP7 cells (MB-MDA-231 cells stably transfected with pSHBG-MP, a construct that encodes a Flag-tagged, mature, processed form of SHBG) constitutively overexpress Flag-tagged SHBG-MP. Total cellular protein extracts were prepared from two MB-MDA-231 transfectant pools and candidate MB-MDA-231-MP clonal cell lines. Western blot analysis, shown above, was used to detect Flag tagged SHBG-MP expression using the Anti-Flag M2 monoclonal antibody (Sigma).

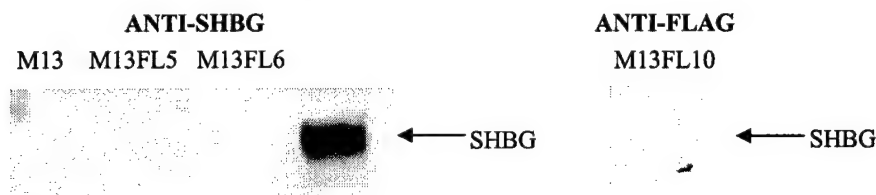


Figure 2B: M13FL10 (MB-MDA-231 cells stably transfected with pSHBG-FL, a construct that encodes a Flag tagged, full length secreted form of SHBG) cells secrete SHBG. M13, M13FL5, M13FL6, and M13FL10 cells were grown in 6 well dishes for 4 days in 1ml of serum-free medium. Cells appeared viable after this period of time. Conditioned medium was collected and centrifuged to remove cells and cellular debris. 30ul aliquots of conditioned medium were analyzed by Western blot, left, using the anti-human SHBG WAK-S102-12-53 antibody. Right, Western blot analysis of M13FL10 using the Anti-Flag M2 monoclonal antibody (no band was apparent). Sample on the right was from the same original Western blot shown as in Figure 2A.

Figure 3. SHBG is alternatively spliced: RT-PCR analysis of MCF-7 cells.

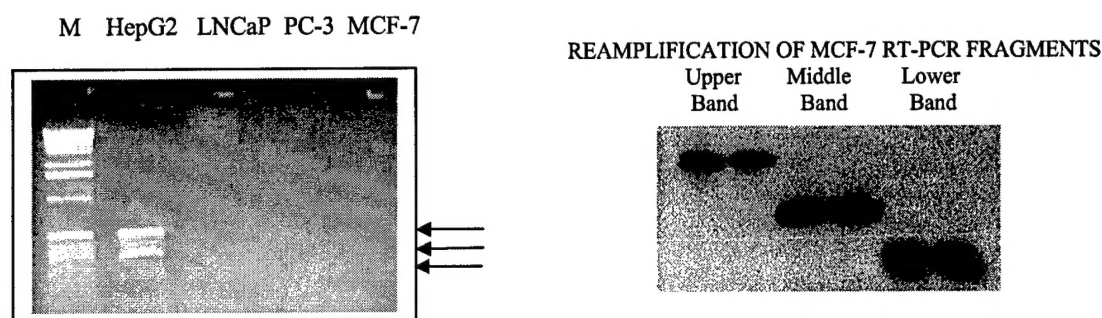


Figure 3. RT-PCR analysis reveals alternative splicing in MCF-7 cells. Left: Total cellular mRNA was prepared from exponentially growing MCF-7, HepG2 (a human hepatic cell line that secretes SHBG and was included as a positive control), LNCaP, and PC3 cells. First strand cDNAs were generated using an oligo dT primer. PCR was then performed using primers predicted to amplify exon 5-8 sequences. Three RT-PCR transcripts were generated in all samples, the predicted 521nt band, and two smaller bands (three light MCF-7 bands (arrows) were visible in the original gel). We have reproduced this alternative splicing pattern three times, using different MCF-7 total cellular RNA preparations. Right: MCF-7 RT-PCR products were electrophoresed alone in a single gel (not shown). DNA from each MCF-7 band was reamplified using the same exon 5 and 8 primers. Duplicate lanes were run for each sample (right), and DNA was extracted from the gel. DNA sequence analysis showed the 521nt band to contain contiguous exon 5-8 sequences. The middle band is missing all of exon 7, and the small band is missing exons 6 and 7. M: DNA size marker.

Figure 4. MCF-7 cell SHBG mRNAs do not contain the hepatically secreted isoform exon 1 sequences.

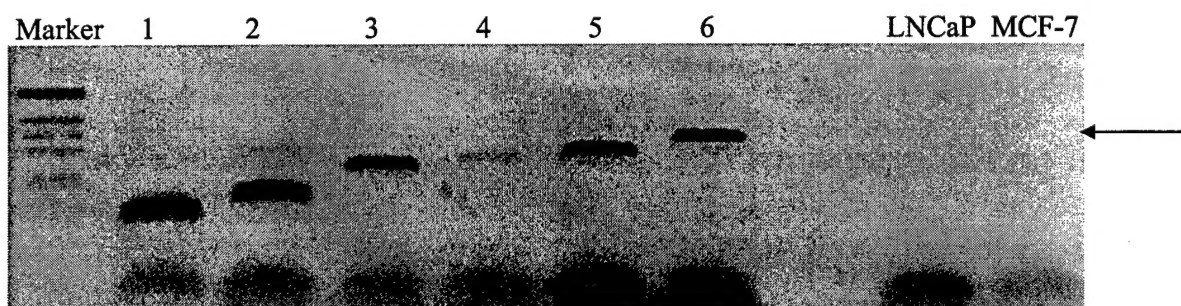
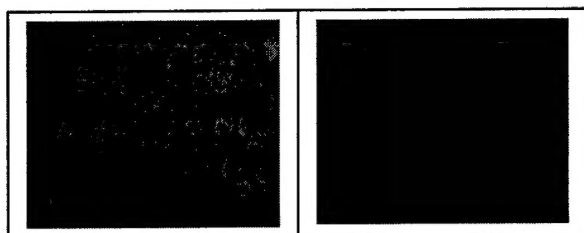
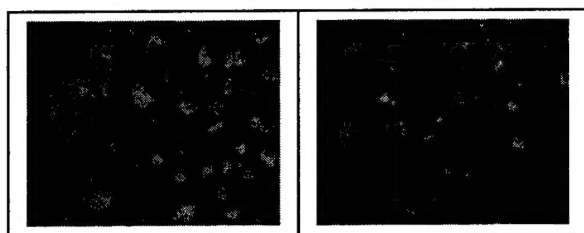


Figure 4. RT-PCR analysis of SHBG exon 1 sequences. The same first strand cDNA samples from Figure 3 were amplified by PCR. Control HepG2 RT-PCR amplifications were performed using a single exon 1 forward primer and six different exon 2 (lanes 1 and 2) or exon 3 (lanes 3-6) reverse primers in order of increasing fragment size. LNCaP and MCF-7 RT-PCR amplifications were performed using the same exon 1 forward and a single exon 3 reverse primer (the same set as in HepG2 lane 6). RT-PCR transcripts of expected sizes are present in all HepG2 amplifications. The exon 1-3 RT-PCR fragment is absent in MCF-7 and LNCaP cells (arrow).

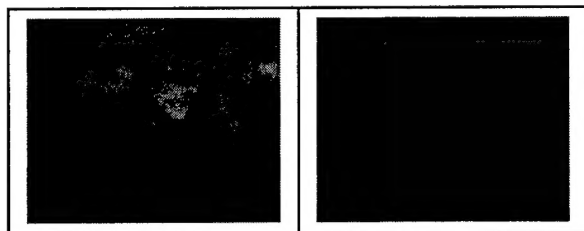
Figure 5. Immunohistochemical detection of SHBG in MCF-7, MD-MBA-231, and 184-B5 cells



MCF-7 cells- IgG1



MD-MBA-231 cells- IgG1 control



C: 184-B5 cells- SHBG

184-B5 cells- IgG1 control

Figure 5. Immunohistochemical detection of SHBG in MCF-7, MD-MBA-231, and 184-B5 cells. Cells were plated on glass slides, fixed, and exposed to a monoclonal antibody (5B2) to SHBG (left panels) or mouse IgG1 (right panels), and developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa-488. Nuclei were stained with the blue dye, DAPI.

Figure 6. SHBG binding to the MCF-7 cell surface is displaced by specific steroids

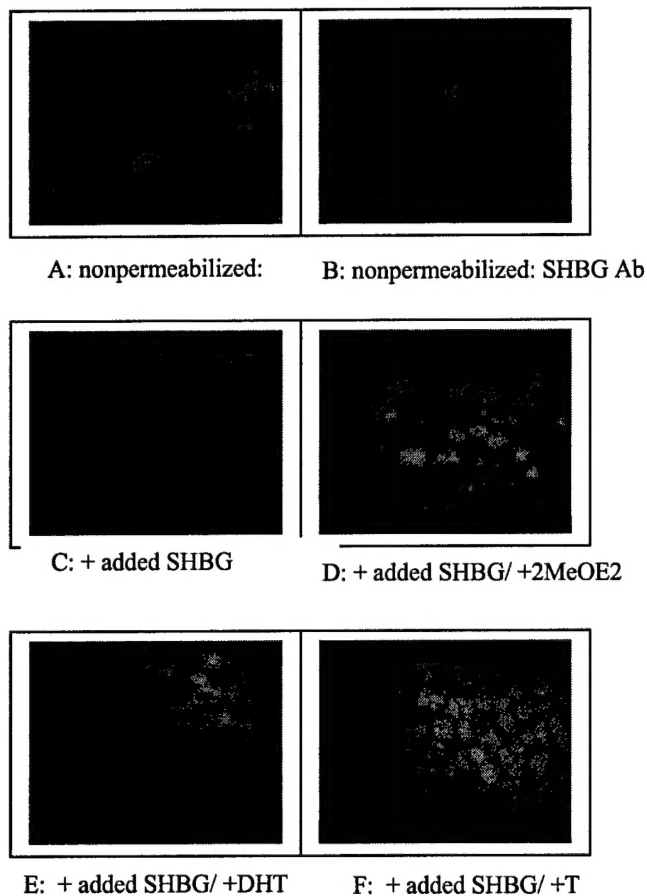


Figure 6. SHBG binding to the MCF-7 cell surface is displaced by steroids that bind to SHBG. MCF-7 cells, grown in serum free medium, were fixed and treated in the absence of the permeabilizing agent, Triton X-100. Cells were incubated in the absence of added SHBG (A, B), in the presence of added SHBG alone (C), or with SHBG and the indicated steroid (D-H). Cells were visualized with a monoclonal antibody (5B2) to SHBG (green) and were counterstained with DAPI (blue). The antibody control was mouse IgG1.

Figure 7. Immunohistochemical analysis of SHBG protein in normal breast and tumor tissue

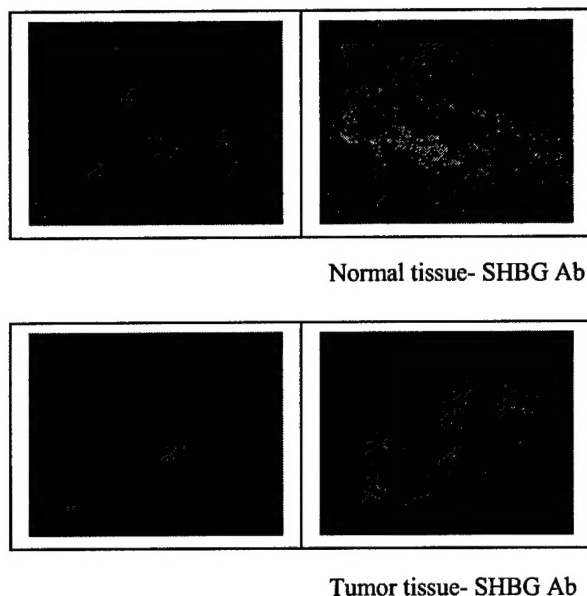


Figure 7. Immunohistochemical analysis of SHBG protein in normal breast and breast tumors. Sections of frozen samples of normal human breast tissue (A), and tumor tissue (B) stained with either IgG1 (control-left panels), or with rabbit anti-SHBG (FITC, green-right panels). Sections were counterstained with the nuclear stain, DAPI (blue).

Figure 8. In situ analysis of SHBG mRNA in normal breast and tumor tissue

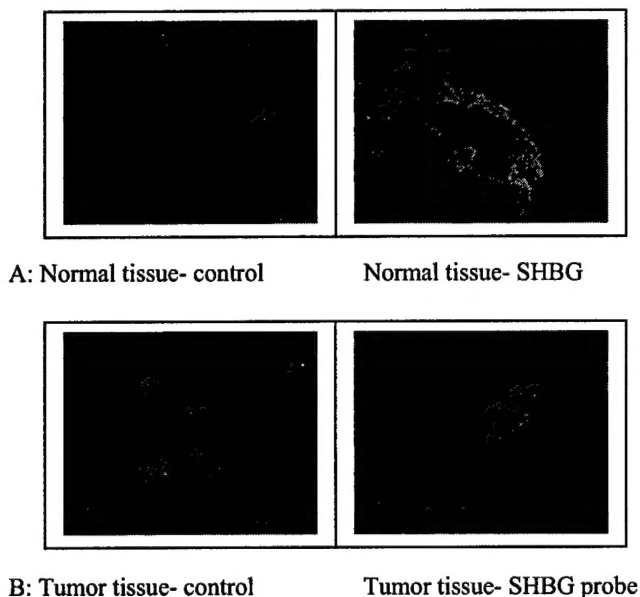


Figure 8. In Situ hybridization of SHBG mRNA in normal breast and tumor tissue. Sections were incubated with a 521 bp (corresponding to the secreted SHBG cDNA fragment from nt.628-1148) SHBG probe. SHBG-FITC probe, green; nuclear counterstain, DAPI, blue.